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Effects of salinity and nitrogen source on growth and lipid production for a wild algal polyculture in produced water media

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ABSTRACT

An algae polyculture (discovered in oil and gas produced water (PW) originating from a production facility in the Permian Basin of southwestern New Mexico) was composed primarily of *Cyanobacterium aponinum*, *Parachlorella kessleri*, with the remaining populations consisting of several species of halotolerant bacteria. The polyculture was tested in PW based media at a variety of different salinities and initial nutrient concentrations to determine the effects of these environmental conditions upon growth and lipid production. The polyculture exhibited growth rates of 46–51 mg ash free dry weight (AFDW)/L/D in PW over a salinity range from 15 to 60 g total dissolved solids (TDS)/L in PW containing nitrate as a nitrogen source. Growth was reduced at higher salinity. Biomass productivity in 60 g TDS/L PW was higher with ammonium as the nitrogen source (47.2–57 mg AFDW/L/D) than nitrate, with the highest growth observed using initial concentrations of 13 mg NH_4-N and 1.7 PO_4-P/L (single day growth of 99 mg AFDW/L/D). Of the conditions tested lipid productivity was greatest (around 12 mg lipids/L/D) at a salinity of 60 g TDS/L after phosphorus depletion (ammonium still present), and the maximum lipid content (48% of AFDW) after ammonium depletion (phosphate still present). Higher salinity, initial ammonium, and phosphate levels enriched for *P. kessleri* over *C. aponinum*, which also resulted in increased lipid content and productivity. Conversion of lipids to fatty acid methyl ester (FAME) created a profile dominated by palmitic (C16:0) and stearic (C18:0) acid and would produce a usable biodiesel. The results indicated that a mixed culture of *C. aponinum*, *P. kessleri,* and other micro fauna is a potential candidate for cultivation in brackish to hypersaline PW based media for the production of biomass and/or biofuels.

1. Introduction

The need to address climate change in recent years has driven the search for carbon-neutral fuels that do not increase atmospheric concentrations of greenhouse gases. Renewable biofuels are one route that has been explored extensively for large-scale production [1]. Algae are an attractive feedstock because of their rapid growth rates compared to traditional crops [2]. Growing algae for biofuels on an industrial scale requires a large volume of water, which is a scarce resource in many areas with sufficient sunlight and land for cultivation, such as the arid southwestern United States [3]. Resource demands for water and nutrients have the potential to put fuel and food production in direct competition. These pressures have increased interest in using wastewaters as algae growth medium, especially if it already contains crucial sources of nitrogen and phosphorus. In addition, algae can remediate wastewater through nutrient removal [4].

One potential source of wastewater for algae cultivation is oilfield produced water (PW) [5]. PW can contain potentially toxic constituents such as metals, volatile organic compounds (VOCs), and radionuclides, and it commonly has salinities well in excess of seawater depending on local geologic conditions [6]. Though PW contains many toxic components that might inhibit algae growth, it also contains a number of nutrients required for cultivation. The crucial nutrient nitrogen is commonly available in the form of ammonium at levels in excess of commercial algae growth media [7,8]. The presence of phosphate has been reported in a number of studies at levels adequate for algae growth (> 1 mg/L PO_4-P) [9] [10,11]. Lastly, important trace elements such as iron, potassium, and manganese are present in sufficient

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quantities to meet algae growth requirements [5]. Nutrient addition can represent 45% of the effective energy input and costs of algae cultivation [12]. The presence of nitrogen and phosphorus in a wastewater medium improves net energy production and return on investment.

A number of different photoautotrophic species have been shown to grow in brackish to saline PW including *Nannochloropsis salina, Dunaliella tertiolecta,* wild eukaryotic, and cyanobacteria strains [5,8,13–15]. The highest PW salinity tested so far in the literature is 41.7 g TDS/L [8], although a large proportion of PW contains dissolved solids well in excess of this value in many basins. In the Permian and Delaware Basins of southeastern New Mexico and west Texas variation in total dissolved solids (TDS) has been measured from 10 to 330 g/L. These values span the range from brackish water to saturated sodium chloride with an average concentration of 154–225 g TDS/L [6]. Similar hypersaline character exists in the Bakken Region of North Dakota and the Marcellus shale of the Eastern United States [7,16]. Initial saline conditions in large volume open high-rate algal ponds (HRAPs) would become increasingly hypersaline over a period of weeks, especially in arid dry climates without dilution by scarce freshwater [17]. If algae are to fully utilize PW, then species and strains that can handle the hypersaline conditions are therefore of great interest.

Previous studies of photoautotrophic growth in PW have focused on single strains. To the best of our knowledge mixed algal-microbial communities have not been studied in produced waters, although polycultures may have improved growth stability over monocultures [18]. In addition, prokaryotes in the polyculture can make essential nutrients like carbon, nitrogen, and phosphorus bioavailable to algae [19]. For economic reasons open pond cultivation will likely be the method for large scale algae cultivation and pure cultures in such systems are likely impossible in this setting. A robust algae consortium that can grow in hypersaline conditions would have advantages over predators and competitors, due to the fact that few other organisms can survive at such high ionic strengths [20].

In this study, a polyculture of microalgae and other prokaryotes was tested in PW at a range of salinities and initial nutrient concentrations. The polyculture was first isolated from PW obtained from the Permian Basin of southeast New Mexico. Preliminary observations indicated it had enhanced salinity tolerance of over 100 g TDS/L. The objective of this study was to determine the biomass and lipid productivity of this unique polyculture in hypersaline conditions representative of those found in PW from a variety of regions. These two parameters are key considerations for biofuels production [21–23]. The composition of the fatty acids, measured as FAME, is also an important consideration if algae lipids are to be used as a feedstock for biofuel production [24,25]. The FAME profiles were analyzed at different salinities. The conditions tested in this study would be representative of those found in PW samples from a variety of regions.

2. Methods

2.1. Analytical methods

2.1.1. Dissolved constituents

The original PW's salinity, cation, anion, and alkalinity were determined as follows. Total dissolved solids (TDS) were determined by four gravimetric measurements (Al104; Metteler Toledo; Columbus, OH, USA) after evaporation of filtered samples [26]. Cation concentrations were measured with inductivity coupled plasma- optical emission spectroscopy on two different samples (Model Optima 5300DV; PerkinElmer; Waltham, MA, USA). The chloride and sulfate content were found for two samples by Ion Chromatography (IC) (Dionex™ ICS-6000 Standard Bore and Microbore HPIC™ System; Thermo Fischer Scientific; Waltham, MA USA). Alkalinity was determined through acid titration to a pH of 8.3 (for carbonate) and 4.5 for (for bicarbonate) [26].

The pH throughout the study was determined using an electrical probe (UB-10; Denver Instruments; Denver, CO, USA). The following constituents were measured in the PW and experimental media spectrophotometrically (DR2700; Hach; Loveland, CO, USA) with commercial reagent sets as per manufacturer's instructions: Chemical oxygen demand (COD) (COD Low Range Test Kit #2125815; Hach), nitrate (Spectroquant Nitrate Test for Seawater #14942; MilliporeSigma; Burlington, MA, USA; which is resistant to interference from other dissolved ions), phosphate (Spectroquant phosphate test kit #14543; MilliporeSigma), and total ammonium (High Range Ammonia Reagent Test Kit # 2606945; Hach). Commonly during experiments for nitrate, ammonium, and phosphate measurements, a composite sample was analyzed with equal proportions of sample from each flask within a triplicate condition.

2.1.2. Biomass

Biomass density was measured using optical density, dry weight (DW), and Ash Free Dry Weight (AFDW) over the course of the study. Optical density (OD) was measured at a wavelength of 680 nm (average peak chlorophyll A absorption) using a DR2700 spectrophotometer (Hach) with deionized water as a blank. An average of three measurements was recorded. DW and AFDW were found using procedures described in Standard Methods [26] as follows: 15–25 mL of algae culture was passed through 0.45 μm glass fiber filters (Pall; Port Washington, NY, USA), and then dried at 105 °C until a constant weight was achieved. Samples were then heated to 550 °C for 1 h to vaporize organics and the mass loss was used to find AFDW. Calibration curves were made to correlate OD680 and AFDW for each experiment and used to determine AFDW in the absence of filtered biomass measurements (Raw data)($R^2 \geq 0.971$). AFDW data points are plotted as an average of OD680 measurements on each flask within a triplicate condition (Converted to AFDW with the second order binomial equation in Raw data). AFDW error bars where shown were found with Excel's standard deviation (SD) function (using the "N-1" method, $n = 3$). Microscopy (BX51; Olympus; Center Valley, PA, USA) was also used to characterize microbial populations and monitor for contamination.

2.1.3. Total lipid concentration and content

The lipid extraction technique from the polyculture biomass was based on chemical solutions and methods presented in Wang and Benning [27]. The biomass in 30–50 mL of culture (one from each flask in a condition) was first concentrated by centrifugation (5804 R; Eppendorf; Hauppauge, NY, USA) at 8000 RPM. The supernatant was removed, and the biomass pellet was stored at −23 °C. The pellet was then thawed, and lipids extracted by adding a solution of methanol, chloroform, and formic acid (20:10:1) in a proportional volume to the biomass (usually 2–4 mL). The mixture was vortexed (945,300; VWR) for approximately 5 min to facilitate cell lysis. Solids in suspension were observed for pigment bleaching which was indicative of cell lysis and lipid dissolution into the solvent. A solution of 0.2 M phosphoric acid and 1 M potassium chloride was then added at half the volume of the methanol, chloroform, formic acid solvent to initiate phase separation and the mixture vortexed. Next to facilitate phase separation, the mixture underwent centrifugation at 8000 RPM for 5 min, and the lower chloroform phase was removed by pipette into 5 mL glass vials. The chloroform phase was then left to evaporate for 48 h leaving behind the extracted lipids. To remove residual salts and other impurities, 1 mL of pure chloroform was then added to each vial to re-dissolve the lipid portion of biomass and then decanted into a fresh glass vial. After an additional 48 h of chloroform evaporation, the lipid extract mass was determined gravimetrically (Average of three measurements), and samples were frozen at −23 °C for later FAME analysis. The final extracts were fully dissolvable in chloroform, and further dissolution/evaporation cycles (with chloroform) did not leave behind any residue. Lipid concentration was found by dividing the mass of the lipids by the initial sample volume. To determine the lipid content of biomass, the lipid concentration was divided by that of the AFDW. Lipid

measurement error bars where shown were found with Excel's SD function (using the "N-1" method, $n = 3$).

2.1.4. Lipid FAME profile

The relative proportions of FAME derived from lipid extracts were determined by gas chromatography mass spectroscopy (GC–MS) following a previously described procedure involving in situ transesterification [28]. Lipid extracts were first re-dissolved in a 2:1 chloroform/methanol solution at a concentration of 1 mg lipid per mL of solution. To accomplish FAME catalysis 300 μL of a 0.6 M potassium hydroxide/methanol solution was added to 250 μL of the dissolved lipid sample. The mixture was placed on shaker table in an incubator at 50 °C for 30 min, after which it was neutralized with 100 μL of glacial acetic acid. 250 μL of hexane (with an internal C23:0 FAME standard) was then added and the top FAME-containing hexane layer was pipetted into sealed sample vials for analysis.

FAME analysis was performed using gas chromatograph (Varian CP-3800; Agilent Technologies: Santa Clara, CA, USA) fitted with a DB-23 column (60 m × .25 μm × .25 μm; Agilent) coupled to a mass spectrometer (Varian 2000, Agilent) scanning from 50 to 500 mass units. The relationship between peak area and concentration of different saturated and unsaturated carbon chain lengths was calibrated against an external standard solution using a serial dilution of 1:10 and a C23:0 internal standard. Peak areas were then used to calculate individual FAME concentration, and the relative proportion determined as the percentage of total FAME observed. Average percent values are plotted in figures and SD found as it was for AFDW and lipids.

2.1.5. DNA sequencing of polyculture

Illumina sequencing of the Polyculture was performed by MR DNA (www.mrdnalab.com, Shallowater, TX, USA). DNA was extracted from algae biomass samples and then sequenced with Illumina using the 23s RNA universal algae primers p23SrV_f1 (5' GGACAGAAAGACCCTAT GAA 3′) and p23SrV_r1 (5' TCAGCCTGTTATCCCTAGAG 3′), which targets a gene encoding for a section of the 23s subunit rRNA occurring in cyanobacteria and plastids [29]. The 16s RNA universal primers 515F (5'GTGCCAGCMGCCGCGGTAA 3′) and 806R (5' GGACTACHV-GGGTWTCTAAT 3′) were used once during the initial polyculture identification to characterize prokaryotes present [30]. A barcode was placed on the forward primer in a 30 cycle polymerase chain reaction (PCR) (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Next, the pooled and purified PCR product were used to prepare the DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA on a MiSeq following the manufacturer's guidelines. Sequence data were processed using the MR DNA analysis pipeline. Briefly, sequences were joined, depleted of barcodes, and sequences with < 150 bp or ambiguous basae calls removed. Sequences were denoised, operational taxonomic units (OTUs) generated and chimeras removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnical Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Experimental methods

2.2.1. Polyculture

The polyculture used in this study was obtained from a continuous batch culture at the Santa Fe Community College Biofuels Center of Excellence (SFCC). The original sample was found in PW from a single oil well in the Permian Basin of southeast New Mexico and obtained from Luke Spangenberg of SFCC.

Table 1
Chemical composition of PW used in this study.

Component	Value
TDS	6.00 ± 0.05 g/L
Sodium	2023 mg/L
Chloride	2352 mg/L
Sulfate	843 mg/L
Bicarbonate	976 mg/L
Carbonate	120 mg/L
Potassium	113.8 mg/L
Magnesium	167 mg/L
Calcium	18.0 mg/L
Lithium	10.5 mg/L
Copper	2.42 mg/L
Iron	Below detection limits
Arsenic	0.386 mg/L
Ammonium	0.1 mg NH_4-N/L
Phosphate	Below detection limits
pH	9.1
COD	6 mg/L

2.2.2. Growth media

The growth medium base in all experiments was either PW obtained from an oil production facility in the Permian Basin of southeast New Mexico, supplemented as described below, or the commercial f/2 medium (University of Texas (UTEX); Austin, TX, USA). The chemical composition of the unaltered PW is listed in Table 1, as determined by the analytical methods described previously. The total alkalinity was 20 mM or approximately 1000 mg/L as $CaCO_3$. Prior to use the PW was filtered with 0.45 μm cellulose acetate filters (Pall). This PW was supplemented with sodium chloride (to vary TDS concentrations in experiments) and nutrients (to vary initial nitrogen and phosphorus concentrations).

2.2.3. Experiments

Three experiments (two salinity tests and one varying nutrients with constant salinity) were conducted to determine the effects of salinity and nutrient concentrations on growth and lipid production, using the PW shown in Table 1. For the two salinity tests, sodium chloride was added to the PW to vary the salinity at the following concentrations 15, 30, 60, 75, 90, 105, 120, and 150 g TDS/L. In the salinity test experiments nitrogen and phosphorus levels were set to approximate the f/2 commercial medium with 13 mg NO_3-N/L and 1.7 mg PO_4-P/L. A trace metal solution (providing iron and other trace metals) was added based on the f/2 medium recipe with the following components in mg/L: 23 $ZnSO_4$-$7H_2O$, 152 $MnSO_4$-H_2O, 7.3 Na_2MoO_4-$2H_2O$, 14 $CoSO_4$-$7H_2O$, 6.8 $CuCl_2$-$2H_2O$, 4.6 Fe(NH4)2(SO4)2-6H2O, and 4.4 Na_2EDTA-$2H_2O$. The pH was then lowered to 8.1 (similar to seawater) with hydrochloric acid.

An additional experiment (nutrient test) was performed changing the nitrogen source to ammonium and testing a higher phosphate concentration holding salinity constant at 60 g TDS/L. Varying initial ammonium concentrations of 13, 30, 50, and 70 mg NH_4-N/L were tested in triplicate. Initial phosphate levels were 1.7 mg PO_4-P/L except for one high phosphorus condition of 8 mg PO_4-P/L with 13 mg NH_4-N/L. Trace metals levels and pH were set identical to the salinity experiments.

To compare polyculture PW growth to a commercial medium a f/2 medium conditions was included in the nutrient test experiment (as a control). Instant Ocean® commercial sea salt (Spectrum Brands, Madison, WI, USA) was used to establish a seawater salinity [31]. 13 mg NO_3-N, 1.7 mg PO_4-P, and 1 mL of trace metal solutions

(described previously) were added per liter, at the same concentrations as for the PW. A vitamin solution of vitamin B_{12}, biotin, and thiamine was added prepared per UTEX instructions. The pH was adjusted to 8.1 with hydrochloric acid, and the entire medium autoclaved prior to inoculation.

2.2.4. Experimental procedures

All experiments were in batch, with triplicate flasks for each experimental condition. 500 mL glass flasks, each containing 250 mL medium, were placed on a shaker table (DR-500e; VWR), at 140 RPM in a 24 °C incubator (307C; Fisher Scientific). Illumination was by four 975 lm fluorescent grow bulbs (BUAG24; Agrosun; Petaluma, CA, USA) with a measured incident intensity of 100 μmol/m^2/s on the table surface and 16-hour light/8-hour dark cycle. Flask positions on the shaker table were randomly changed to avoid biases due to differences in illumination. The experiment was continued into the stationary phase of growth with the total time ranging from 17 to 40 days. Periodically (usually every 1–2 days), samples were removed for biomass and media composition measurements. For nutrient concentrations (nitrate, ammonium, and phosphate), a composite sample taken in equal proportions, from each triplicate flask in the condition was analyzed. The composite sample was measured using spectrophotometric methods described previously. When individual flask volumes fell below 100 mL the triplicate condition was combined into one flask (none before day 16).

All flasks were inoculated from cultures that were previously acclimatized at the salinity to be tested. Algae cultures used as inoculates were in linear growth phase. Algae cells were separated from the previous medium by centrifugation at 3500 RPM prior to addition to the media. Sufficient inoculum was added to provide initial biomass concentrations in the range 0.04–0.05 g AFDW/L. Cultures were checked microscopically for live cells and contamination for the duration of each experiment.

3. Results and discussion

3.1. Pre-experiment DNA sequencing results

The initial 23s primer set sequencing results (designed to identify both eukaryotic and prokaryotic algae) of the polyculture (immediately prior to experimentation) were dominated (> 99%) by three different sequences (Fig. 1). The most common group detected with the 23s primer was *Cyanobacterium aponinum* (63% of all OTUs) previously described growing in thermal spring water in Italy [32] and saline oil and gas wastewater in Oman [33]. Cell morphology from Moro et al. [32] matches microscopic images of the polyculture showing diplo rod and oval forms 1–2 μm in length. Previously reported halotolerance for *C. aponinum* matches observations in this study [33]. *C. aponinum* has been suggested as a candidate for biofuel production with a peak lipid content of up to 45% [34]. The uncultured clone comprising 25% of the sample has yet to be identified but was 99% similar with a sequence from cyanobacterial mats [35]. The closest identified match was to *Phycisphaera mikurensis* (with an 81% homology). The 23s primer used also amplifies some non-photosynthetic prokaryotes and the uncultured clone may fall in this category. The third most prevalent sequence *Parachlorella kessleri* has a spherical morphology with a cell size (1–5 μm) similar to cells observed in the polyculture. It is also considered a biofuel candidate with an oil content up to 60% [36] and has a high growth rate [37]. To the best of the authors knowledge no other studies have yet been found testing either species in PW. Observed algae cells in the polyculture either fall in the diplo cyanobacterium form (suspected *C. aponinum*) or spherical eukaryote (suspected *P. kessleri*). The 16s universal prokaryotic primer identified a number of halotolerant bacteria species and *Cyanobacterium aponinum* (Fig.1).

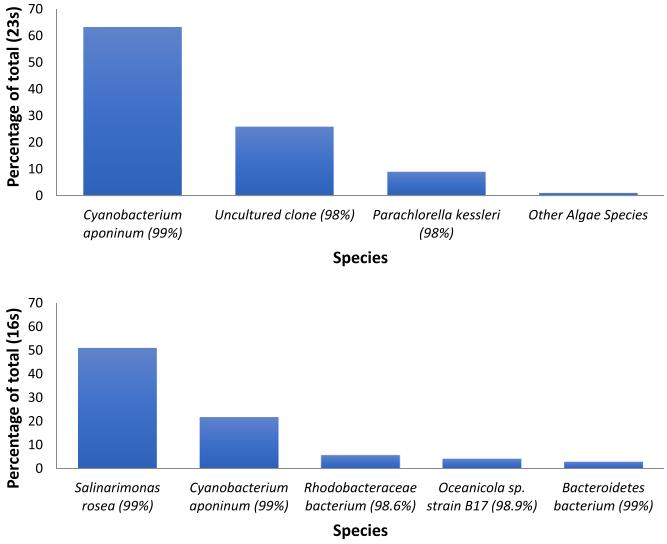

Fig. 1. Illumina Sequencing Results for the Polyculture with 23s and 16s primers. The percent homology is in parentheses.

3.2. Effects of variable salinity on growth, lipids, and populations

3.2.1. Growth in PW at a range of salinities (salinity test)

In the first salinity test experiment, the polyculture exhibited similar growth profiles at 15, 30, and 60 g TDS/L (Fig. 2), with average growth rates between day 2 and 7 range from 46 to 51 mg AFDW/L/D in these cultures. At salinities of 75 and 90 g TDS/L growth was reduced at 14.0 and 6.4 mg AFDW/L/D respectively over the same time frame (P value < 0.01). Little to no biomass increase was observed in flasks with salinities ≥105 g TDS/L. Cell forms similar to *P. kessleri* and *C. aponinum* were observed in all cultures. After three weeks in 120 g TDS/L PW, dilution to 60 g TDS/L was able to restart growth. The results suggest the *P. kessleri* and *C. aponinum* dominated polyculture may be suitable for growth in highly saline produced waters, with an upper salinity tolerance at or just above 60 g TDS/L.

The growth rate of 46 mg/L/D in hypersaline, 60 g TDS/L PW in this study was comparable to results for *Nannochloropsis salina* and *Dunaliella tertiolecta* in shale flowback water at a lower salinity (41.7 g TDS/L) in 250 mL flasks [8]. Compared to the polyculture, *N. salina* exhibited substantial growth inhibition in > 46 practical salinity unit (PSU) media (a PSU is approximately equal to g TDS/L) [38]. The polyculture's higher halotolerance (up to 60 g TDS/L) may make it a superior candidate to *N. salina* for growth in hypersaline produced waters.

Illumina DNA sequencing was performed on samples from Day 16 of the experiment for flasks with salinities up to 90 g TDS/L, using the 23s primer pair (Fig. 3). *C. aponinum* and *P. kessleri* represented (> 89%) of the identified OTUs. *C. aponinum* was more abundant in the 15 and 30 g TDS/L conditions while *P. kessleri* in the hypersaline. The results suggest a greater halotolerance for *P. kessleri*, though both species coexist over a broad range of salinity. While it is probable both *P. kessleri* and *C. aponinum* are in direct competition for light and nutrients a symbiotic relationship could exist. *Loktanella vestfoldensis* and *Aquiflexum balticum* are non-photosynthetic bacterium that happened to be successfully amplified by the “algae” targeted 23s primer, and have been found with microalgae in marine environments [39,40].

3.2.2. Lipid production in PW at a range of salinities

Polyculture lipid production varied with salinity. Although the growth rates for the 15, 30, and 60 g TDS/L flasks were similar (Fig. 2), lipid enrichment post nutrient depletion was only displayed by the 60 g TDS/L PW with a peak content of 31% of AFDW (Fig. 4). Increased lipid content after nutrient depletion has been reported in a number of

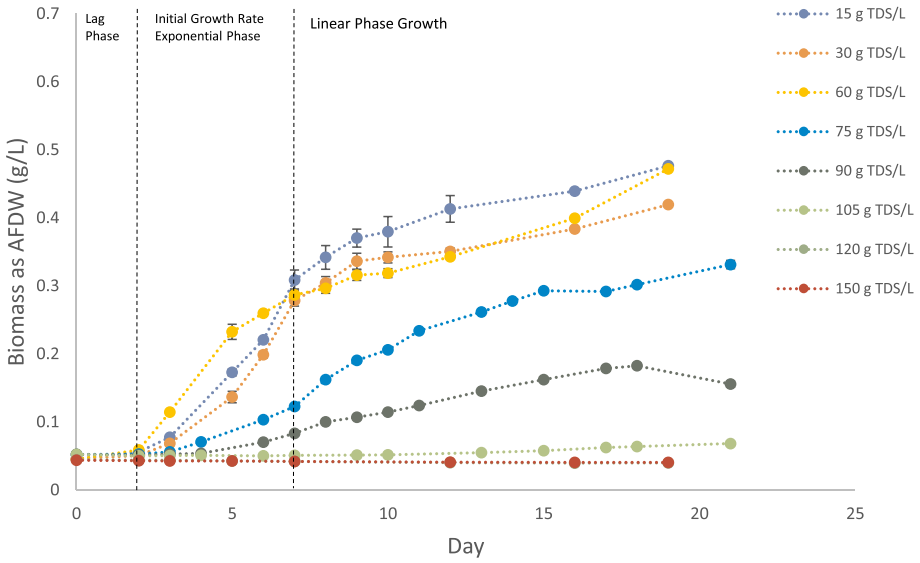

Fig. 2. AFDW measurements of the polyculture in PW at a variety of different salinities (Salinity Test). Error bars represent one SD (n = 3) of measurements from each triplicate flask within a condition, which often are under the data point. Single measurements after day 13 for 15, 30, and 60 g TDS/L.

studies [21,36,41]. Salinity stress has also been shown to yield lipid enrichment with microalgae [42]. Specifically for *Chlorella vulgaris,* which is in the same family as *P. kessleri* [43]. Lipid fractions post nutrient depletion were even greater in higher salinity conditions of 75 and 90 mg TDS/L, with maximum lipid content of 38% and 40%, respectively (supplemental). Continued polyculture cultivation in a nutrient depleted, hypersaline PW based media appears to be an effective strategy to boost polyculture lipid content.

When lipid productivity is considered, rates were higher in the salinity condition of 60 g TDS/L (9 mg/L/D) ($P < 0.01$) because of higher growth (when compared to 75 and 90 g TDS/L) and higher lipid content (when compared to 15 and 30 g TDS/L) (Table 2). Assuming high lipid productivity is desired for the utilized biofuel conversion process, TDS in the upper range of the polyculture's tolerance for high growth (at or near 60 g TDS/L) would maximize lipid production. The hypersaline salinity levels of PW could likely enhance polyculture lipid yields for transesterification to biodiesel.

3.2.3. Polyculture FAME profile

The FAME composition derived from polyculture lipids was dominated by the saturated fatty acid chains palmitic (C16:0) and stearic (C18:0) (Fig. 5). Unsaturated chains never exceeded 25% of the total in 15, 30, and 60 g TDS/L PW (The lower growth, higher salinity conditions were not analyzed). The high proportion of C16 and C18 FAME show the suitability of the polyculture lipids for transesterification to biodiesel. Based on these profiles the cetane number (a measure of flash point) would range from 75 to 84, well in excess of the minimum value of 47 cited for biodiesel [8]. Saturated carbon chains also have a higher oxidative stability [25] and resistance to bio-deterioration [44]. These results indicate the polyculture would yield a consistent FAME profile for transesterification to biodiesel or hydrothermal liquefaction to biocrude. Whether FAME was created from triglycerides or phospholipids was not determined in this study. *P. kessleri* has been shown previously to accumulate a high proportion of neutral lipids under nutrient stress conditions [36], and if also true of the polyculture would improve the suitability for conversion to biodiesel.

3.3. Effects of nutrient types and concentrations on growth under hypersaline conditions

3.3.1. Effects of nitrogen source, higher phosphate, and ammonium on growth (nutrient test)

In a separate set of experiments, the effects of nitrate versus ammonium as nitrogen source and the effects of phosphorus concentration

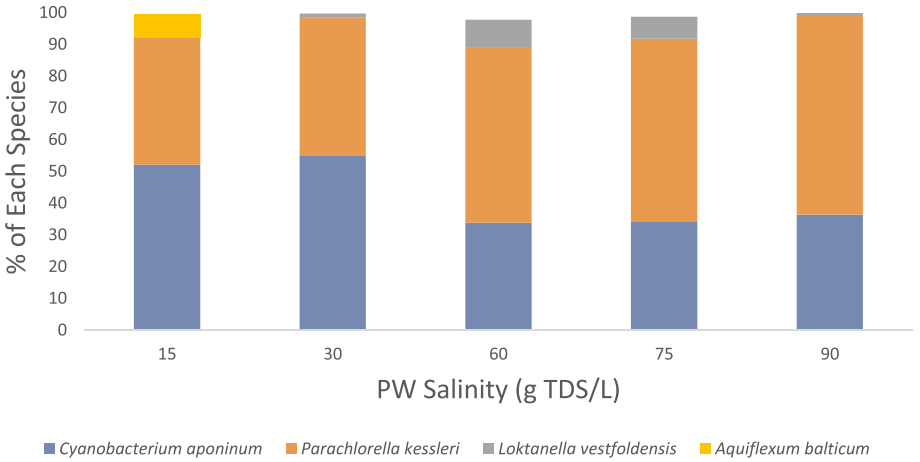

Fig. 3. Illumina DNA Sequencing results for the polyculture in PW at a variety of salinities. Only the percentage of *C. aponinum*, *P. kessleri*, *Loktanella vestfoldensis*, and *Aquiflexum balticum* are shown. The remaining species are composed of non-photosynthetic prokaryotes.

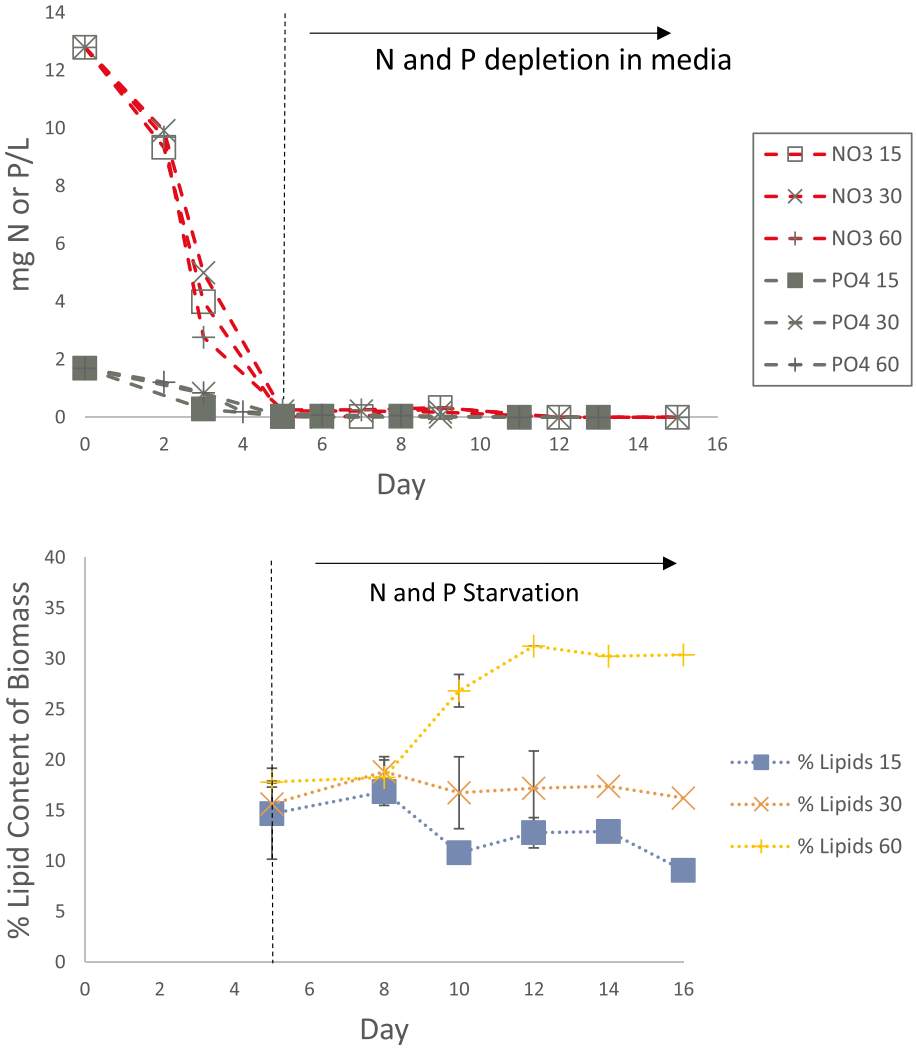

Fig. 4. Nitrate and phosphate concentration (top) and lipid content (bottom) for the 15, 30, & 60 g TDS/L PW conditions. NO_3 and PO_4 values are from composite samples (see methods). Lipid content error bars represent one SD (n = 3) of measurements from each triplicate flask within a condition. Single measurements after Day 13.

were tested to assess their influence on growth and lipid production in 60 g TDS/L PW based media. Biomass increased faster on ammonium compared to nitrate ($P < 0.01$) (Fig. 6a) at the same initial nitrogen concentration of 13 mg N/L. The largest single day increase of 99 mg AFDW/L/D was measured with this initial concentration of ammonium. Algae cells must reduce nitrate nitrogen in order to utilize it, but this is not the case for ammonium [45,46]. This may result in a higher energy requirement for utilization of nitrogen from nitrate over ammonium, therefore decreasing the growth rate.

Opposite expectations, higher initial phosphate concentrations (8 vs. 1.7 mg PO_4-P/L) did not result in increased growth and produced the opposite effect (Fig. 6b). After day 3, the low P medium had a higher growth rate and final biomass concentration ($P < 0.01$). It is possible depletion of both ammonium and phosphate stimulated biomass accumulation between day 4 and 5 for the low P condition. The initial N/P ratio in the low P condition was around 17:1, while it was 4:1 in the high P. A ratio of 17:1 is near the Redfield value for marine phytoplankton [19], which is linked to higher growth rates [47]. Algae are known to store phosphorus primarily as polyphosphate in the cell, which requires energy, for later anabolism [48]. It is possible that at the higher phosphorus concentration, increased polyphosphate storage diverted energy from growth, thereby decreasing the rate of biomass accumulation. The practical implications are that nutrient levels close to 13 mg NH4-N/L and low phosphate concentrations of 1–2 mg PO4-P/L may be ideal for cultivation of the polyculture in hypersaline PW. This level of phosphate has been measured in PW and might eliminate the necessity for its addition.

Early growth rates (between day 2 and 7) for the different initial nutrient levels tested indicated that the lowest ammonium and phosphate values had the highest productivity (Table 3). Growth rates decreased from 57 to 47 mg/L/D with increasing initial concentrations of 13, 30, 50, and 70 mg NH_4-N/L. High free ammonia has been reported

Table 2
Lipid productivity at different salinities (Calculated on Day 12).

PW salinity (g TDS/L)	15	30	60	75	90
Lipid production[a] (mg/L/D)	4.45 ± 0.11	5.1 ± 0.9	9.03 ± 0.01	3.9 ± 0.4	2.5 ± 0.2

[a] Results are presented as mean and ± one SD (n = 3) of measurements from each triplicate flask within a condition.

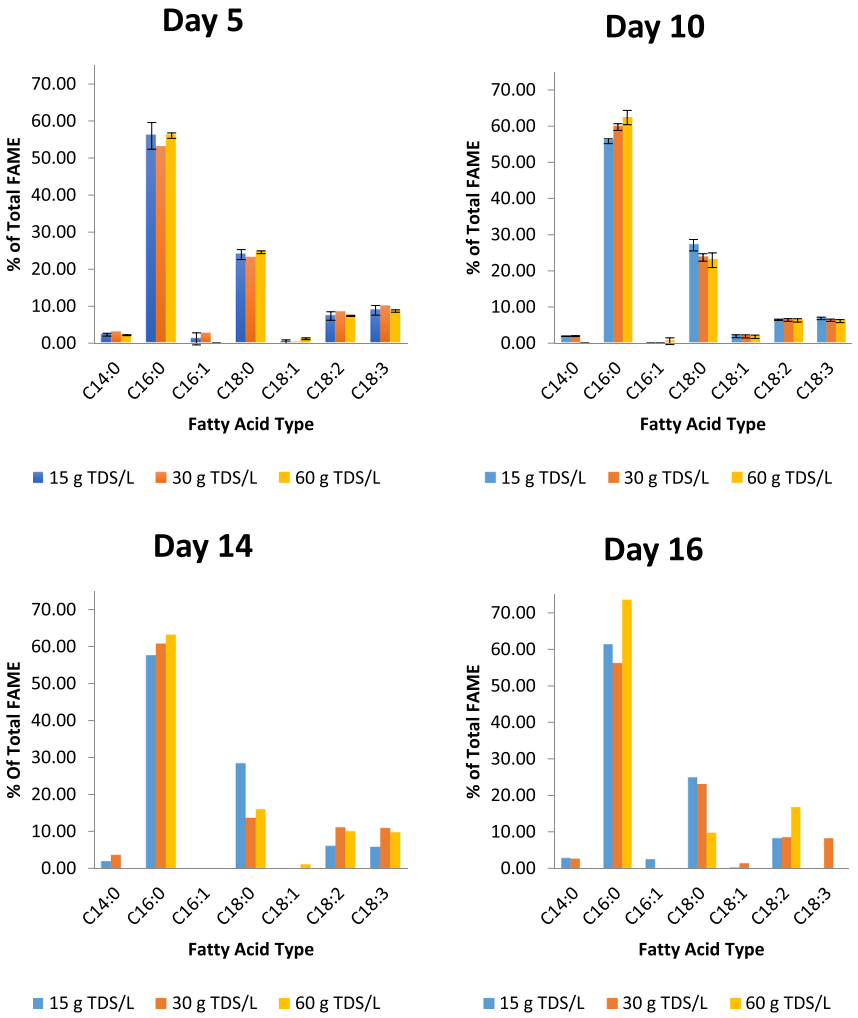

Fig. 5. Polyculture FAME profile over the course of the experiment for the 15, 30, and 60 g TDS/L Conditions. Error bars represent one SD ($n = 3$) of measurements from each triplicate flask within a condition. Only single measurements were made on day 14 and 16 from a combined flask.

to inhibit and even prevent algal growth [49–51], and free ammonia toxicity can occur at levels as low as 2.3 mg NH_3-N/L [52]. The pH of the media increased from 8.1 to over 10 during polyculture growth, which would shift the speciation to free ammonia (pKa = 9.27 for the NH_4^+/NH_3 acid/base pair at the constant temperature of 24 °C used in experiments [53]). With relation to polyculture cultivation in PW, total ammonium levels near 13 mg NH_4-N/L may produce faster growth than higher concentrations (levels above 400 mg NH_4/L have been reported [7]). High ionic strength and colder temperatures [53] increase the pKa for ammonium/ammonia [53,54], which would lower the fraction of free ammonia within the pH range measured in this study's experiments. Similar conditions in outdoor cultivation ponds would likely result in less free ammonia induced inhibition.

The measured growth rates in this study demonstrate the feasibility of growing the polyculture in PW up to 60 g TDS/L. Direct comparison to other studies is always a challenge due to variation in illumination, nutrient levels, gas exchange, reactor type, and other parameters. Results from hypersaline PW flask tests were comparable to other efforts in oilfield waste water [8,13,14]. Biomass productivities of 50–100 mg/L/D match with low to midrange performance of high rate algal ponds (equivalent to approximately 10–20 g/m^2/D) [2], and polyculture performance could likely be increased through the optimization of growth parameters. When growth rates in PW are compared to the commercial f/2 medium, the polyculture displayed lower initial rates in the conventional f/2 than all PW conditions ≤60 g TDS/L. It is possible that this can be attributed to the lower alkalinity of a seawater-based f/2 medium (approximately 2 mM vs. 20 mM). High alkalinity PW based media would likely yield higher growth rates than that from seawater.

3.3.2. *Sequencing results for different nutrient conditions (nutrient test)*

DNA sequencing was performed on samples from days 8 and 19 for the 13 NH4 PW, high P PW, 70 NH4 PW, and f/2 media cultures (Fig. 7). The roughly even split between *P. kessleri* and *C.* aponinum for the inoculate was largely maintained in the highest growth 13 mg NH_4-N and 1.7 mg PO_4-P/L condition on day 8 and day 19 of the experiment. In the PW conditions of high ammonium (70 mg NH_4-N/L) and phosphate (8 mg PO_4-P/L), the majority of OTUs shifted toward *P. kessleri*, and its dominance became more pronounced by day 19. The DNA results matched what was observed microscopically. *P. kessleri* could likely have a greater tolerance to free ammonia, ability to utilize ammonium, and ability to outcompete *C. aponinum* at higher phosphate levels.

The results indicate for the highest biomass productivity in PW conditions favorable for both *P. kessleri* and C. *aponinum* should be considered. Higher ammonium and phosphate levels that inhibit *C. aponinum* lowered initial growth rates between day 2 and 7. The predominance of *C. aponinum* in the f/2 media with approximately one half the growth rate suggests conditions in the PW that also stimulate *P. kessleri* are desirable (possibly higher alkalinity). Conditions that shift the balance between *P. kessleri* and *C. aponinum* might also be relevant for the highest fuel conversion yields depending on the requirements of the exact method.

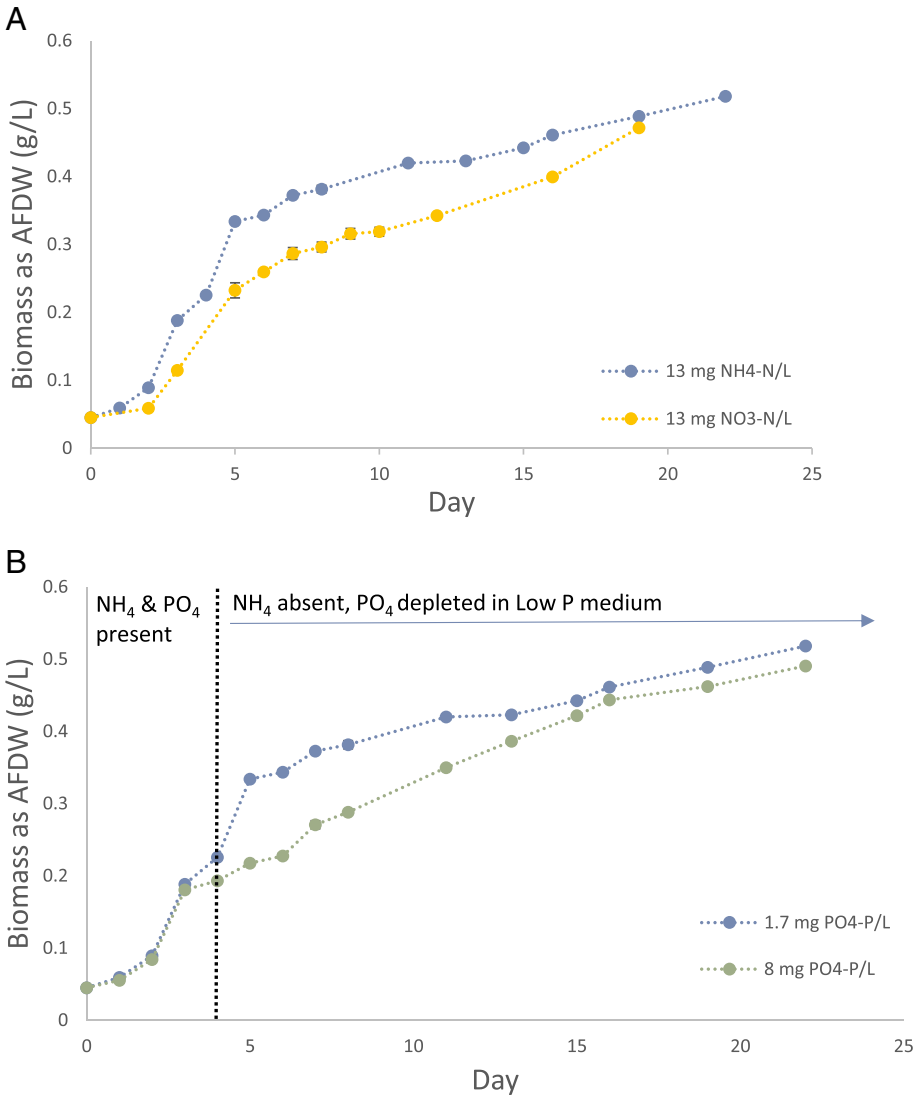

Fig. 6. a: Biomass measurements in 60 g TDS/L PW at initial nutrient concentrations of 13 mg NO_3-N & 1.7 mg PO_4-P/L (NO3) and 13 mg NH_4-N & 1.7 mg PO_4-P/L (NH4). AFDW error bars are one SD (n = 3) and often lie within a data point. AFDW are single measurements after day 16.
b: Biomass measurements in 60 g TDS/L PW at initial nutrient concentrations of 13 mg NH_4-N & 1.7 mg PO_4-P/L (Low P) and 13 mg NH_4-N & 8 mg PO_4-P/L (High P). After day 4 NH_4 was depleted in both conditions, while PO_4 was present in the high P condition but below detection limits in the low P. AFDW error bars are one SD (n = 3) and often lie within a data point. AFDW are single measurements after day 16.

Table 3
Growth rates for the Polyculture in 60 g TDS/L PW media and the f/2 medium at different initial ammonium and phosphate concentrations.

Initial ammonium concentration[a] (mg NH4-N/L, mg PO4-P/L)	Initial growth rate[a] (Day 2–7) (mg AFDW/L/D)
13 mg NH4-N, 1.7 mg PO4-P/L (Low P)	57 ± 1
30 mg NH4-N, 1.7 mg PO4-P/L	55.1 ± 0.2
50 mg NH4-N, 1.7 mg PO4-P/L	50 ± 1
70 mg NH4-N, 1.7 mg PO4-P/L	47.2 ± 0.3
13 mg NH4-N, 8 mg PO4-P/L (High P)	38 ± 1
13 mg NO3-N, 1.7 mg PO4-P/L	46 ± 5
13 mg NO3-N, 1.7 mg PO4-P/L (f/2 medium)	21 ± 2.

[a] Results are presented as mean and ± one SD (n = 3) of measurements from each triplicate flask within a condition.

3.3.3. Lipid productivity and content at a range of initial nutrient concentrations

In this study the polyculture exhibited the highest lipid content after phosphorus or nitrogen depletion of the 60 g TDS/L PW media. The polyculture maximum lipid content of 48% in the High P (after ammonium depletion) and 41% in the 70 mg NH_4-N/L conditions (after phosphate depletion) were greater (P value < 0.05) compared to approximately 30% for the faster growing low ammonium and phosphate flasks (after depletion of both) (Table 4). Peak lipid content was also around 30% when using 13 mg NO_3-N and 1.7 mg PO_4-P/L at the same salinity (after depletion of both). *P. kessleri* represented a greater fraction of the OTUs in the high P and 70 mg NH_4-N/L initial conditions. *P. kessleri* has been previously reported to produce the highest lipid fraction under limitation of one nutrient with nitrogen producing the highest percentage [36]. Stress by only one nutrient might have initiated a lipid enrichment response, while cellular metabolism was not degraded to the point of preventing lipid accumulation. Polyphosphate itself stores energy resulting in increased energy reserves [19], which might have caused algae in the culture to allocate a greater portion of fixed carbon to lipids.

The higher lipid content of the High P and higher ammonium conditions tested resulted in greater lipid productivity. At higher initial ammonium levels of 50 and 70 mg NH_4-N/L (after phosphate depletion) the maximum lipid productivity was approximately 12 mg/L/D and in the High P (after ammonium depletion) close to 11 mg/L/D. This was larger (P value < 0.05) than the 8–10 mg/L/D values measured for lower nitrogen concentrations of 13 mg N/L and 1.7 mg PO_4-P/L for nitrate and ammonium (after depletion of nitrogen and phosphorus). While the lower initial concentrations of ammonium and phosphate resulted in the highest growth rates in this study, lipid productivity would possibly be maximized during cultivation by ensuring the limitation of only one of these nutrients after peak biomass density is

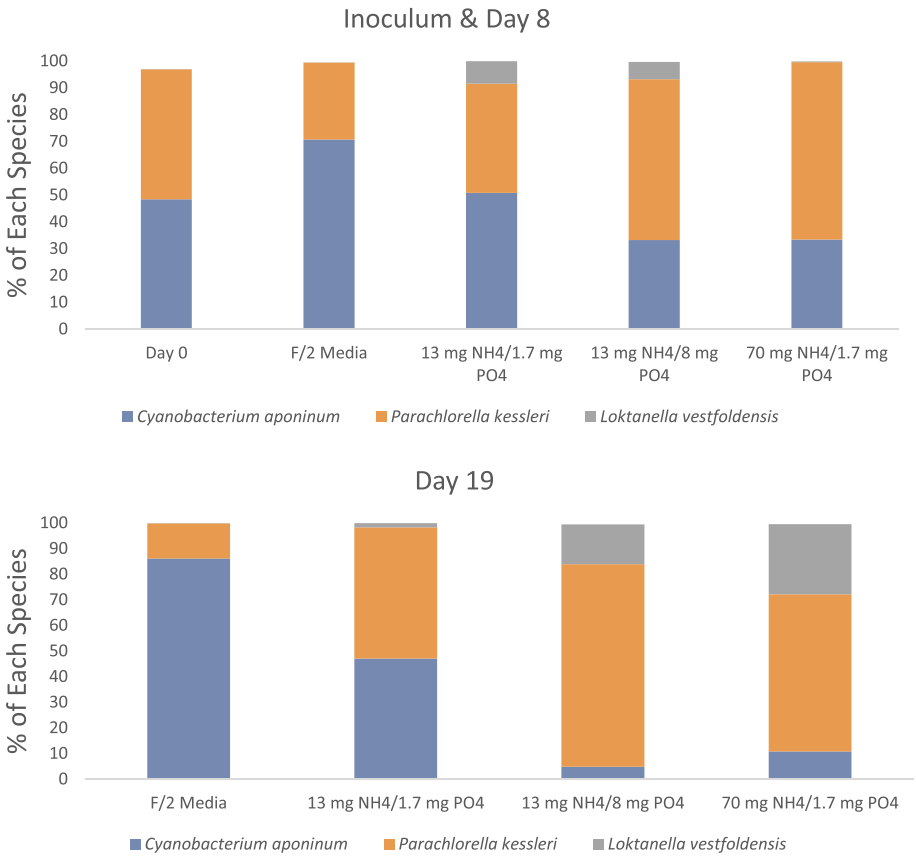

Fig. 7. Relative abundance of each algae species in different initial concentrations of PW and the f/2 seawater medium. The day 0 inoculum is included. Only the percentages of *C. aponinum*, *P. kessleri*, and *Loktanella vestfoldensis* are shown. The remaining species are composed of non-photosynthetic prokaryotes.

Table 4
Maximum lipid production rate and content at different initial ammonium and phosphate concentrations in 60 g TDS/L PW media and the f/2 medium.

Initial nutrient concentrations (mg NH4-N/L, mg PO4-P/L)	Lipid production rate[a] (mg total lipids/L/D)	Lipid content[a] (% total lipids/AFDW)
13 mg NH4-N, 1.7 mg PO4-P/L	9.9 ± 0.8	31 ± 5
30 mg NH4-N, 1.7 mg PO4-P/L	9.7 ± 0.7	27 ± 3
50 mg NH4-N, 1.7 mg PO4-P/L	12.0 ± 0.4	40 ± 3
70 mg NH4-N, 1.7 mg PO4-P/L	11.8 ± 0.8	41 ± 4
13 mg NH4-N, 8 mg PO4-P/L	11.0 ± 0.9	48 ± 5
13 mg NO3-N, 1.7 mg PO4-P/L	9.03 ± 0.11	31.01 ± 0.10
13 mg NO3-N, 1.7 mg PO4-P/L (f/2 medium)	3.8 ± 0.3	13 ± 3

[a] Results are presented as mean and ± one SD (n = 3) of measurements from each triplicate flask within a condition.

reached in the algal pond.

Hypersaline PW at or near 60 g TDS/L with an increased alkalinity over seawater would likely provide enhanced lipid productivity and content for biofuel production. The polyculture in the f/2 medium (30 mg TDS/L) yielded significantly lower lipid productivity (P value < 0.01) and content than the cultures grown in the 60 g TDS/L hypersaline PW in this study. The f/2 and the 30 g TDS/L PW media are approximately at the same salinity, and both exhibited decreased lipid content relative to measurements in the hypersaline conditions of 60 g TDS/L. It is possible the higher alkalinity of PW likely caused the increased growth and lipid content exhibited in this study over the f/2 media at around the same salinity (30–35 g TDS/L) resulting in the higher lipid productivity (5.1 ± 0.9 vs. 3.8 ± 0.3 mg/L/D). In general, higher ammonium, phosphate, and salinity in this study lead to conditions that favored *P. kessleri* and higher lipid content suggesting it as a more oleaginous species that *C. aponinum*.

4. Conclusion

Based on the results of this study, the polyculture (and its component algae species) are potential candidates for biofuel production in PW. Growth remained consistent (46–51 mg/L/Day) over a wide range of salinity from 15 to 60 g TDS/L in PW media using nitrate. A higher biomass productivity was achieved at the 60 g TDS/L salinity using ammonium (57 mg AFDW/L/D), which is likely already present in PW, can be added with wastewater, and/or can be recycled from biofuel production such as anaerobic digestion. PW already possessing the crucial nitrogen and phosphorus nutrients could reduce or eliminate this considerable cultivation cost.

The 60 g TDS/L upper bound of growth tolerance for the polyculture would allow a large percentage of PW to be considered as a growth media. The polycultures tolerance of high salinity, free ammonia, and pH (up to 10.8) would be beneficial in reducing competing and

predatory organisms in outdoor ponds and would allow it to thrive in ammonium-rich PW. Free ammonia growth inhibition to the polyculture could be countered by dilution with a lower ammonium PW source or by adding fresh media to that which has already been depleted. The lower phosphate levels of 1.7 mg PO_4-P/L could be maintained by similar methods. Conditions that favor *P. kessleri* would likely maximize lipid productivity. Future experiments will test the polyculture in outdoor raceway ponds to better determine its suitability for large scale biofuel production.

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References

[1] S. Mobin, F. Alam, Biofuel production from algae utilizing wastewater, 19th Australas. Fluid Mech. Conf, 2014, p. 7.
[2] J.A. Posada, L.B. Brentner, A. Ramirez, M.K. Patel, Conceptual design of sustainable integrated microalgae biorefineries: parametric analysis of energy use, greenhouse gas emissions and techno-economics, Algal Res. 17 (2016) 113–131, https://doi.org/10.1016/j.algal.2016.04.022.
[3] R. Pate, G. Klise, B. Wu, Resource demand implications for US algae biofuels production scale-up, Appl. Energy 88 (2011) 3377–3388, https://doi.org/10.1016/j.apenergy.2011.04.023.
[4] N. Abdel-Raouf, A.A. Al-Homaidan, I.B.M. Ibraheem, Microalgae and wastewater treatment, Saudi J. Biol. Sci. 19 (2012) 257–275, https://doi.org/10.1016/j.sjbs.2012.04.005.
[5] E.J. Sullivan Graham, C.A. Dean, T.M. Yoshida, S.N. Twary, M. Teshima, M.A. Alvarez, T. Zidenga, J.M. Heikoop, G.B. Perkins, T.A. Rahn, G.L. Wagner, P.M. Laur, Oil and gas produced water as a growth medium for microalgae cultivation: a review and feasibility analysis, Algal Res. 24 (2017) 492–504, https://doi.org/10.1016/j.algal.2017.01.009.
[6] B. Chadhaury, S. Willman, K. Caroll, Spatial Variability and Geochemistry of Produced Water in Southeastern New Mexico, USA, (2016).
[7] J.S. Harkness, G.S. Dwyer, N.R. Warner, K.M. Parker, W.A. Mitch, A. Vengosh, Iodide, bromide, and ammonium in hydraulic fracturing and oil and gas wastewaters: environmental implications, Environ. Sci. Technol. 49 (2015) 1955–1963, https://doi.org/10.1021/es504654n.
[8] R. Racharaks, X. Ge, Y. Li, Cultivation of marine microalgae using shale gas flowback water and anaerobic digestion effluent as the cultivation medium, Bioresour. Technol. 191 (2015) 146–156, https://doi.org/10.1016/j.biortech.2015.04.065.
[9] A. Fakhru'l-Razi, A. Pendashteh, Z.Z. Abidin, L.C. Abdullah, D.R. Biak, S.S. Madaeni, Application of membrane-coupled sequencing batch reactor for oilfield produced water recycle and beneficial re-use, Bioresour. Technol. 101 (2010) 6942–6949, https://doi.org/10.1016/j.biortech.2010.04.005.
[10] S.M. Riley, J.M.S. Oliveira, J. Regnery, T.Y. Cath, Hybrid membrane bio-systems for sustainable treatment of oil and gas produced water and fracturing flowback water, Sep. Purif. Technol. (2016) 297–311, https://doi.org/10.1016/j.seppur.2016.07.008.
[11] A. Pendashteh, A. Luqman Chuah, A. Fakhru'l-Razi, S. Siavash Madaeni, Z. Abidin, D. Radiah, Evaluation of membrane bioreactor for hypersaline oily wastewater treatment, Process. Saf. Environ. Prot. 90 (2012), https://doi.org/10.1016/j.psep.2011.07.006.
[12] H.C. Greenwell, L.M.L. Laurens, R.J. Shields, R.W. Lovitt, K.J. Flynn, Placing microalgae on the biofuels priority list: a review of the technological challenges, J. R. Soc. Interface 7 (2010) 703–726, https://doi.org/10.1098/rsif.2009.0322.
[13] V. Aravinthan, D. Harrington, Coal seam gas water as a medium to grow *Dunalliella tertiolecta* microalgae for lipid extraction, Desalin. Water Treat. 52 (2014) 947–958, https://doi.org/10.1080/19443994.2013.827295.
[14] L.H. Hodgskiss, J. Nagy, E.P. Barnhart, A.B. Cunningham, M.W. Fields, Cultivation of a native alga for biomass and biofuel accumulation in coal bed methane production water, Algal Res. 19 (2016) 63–68, https://doi.org/10.1016/j.algal.2016.07.014.
[15] J.L. Wood, C.D. Miller, R.C. Sims, J.Y. Takemoto, Biomass and phycocyanin production from cyanobacteria dominated biofilm reactors cultured using oilfield and natural gas extraction produced water, Algal Res. 11 (2015) 165–168, https://doi.org/10.1016/j.algal.2015.06.015.
[16] N.E. Lauer, J.S. Harkness, A. Vengosh, Brine spills associated with unconventional oil development in North Dakota, Environ. Sci. Technol. 50 (2016) 5389–5397, https://doi.org/10.1021/acs.est.5b06349.
[17] T. Ishika, N.R. Moheimani, P.A. Bahri, Sustainable saline microalgae co-cultivation for biofuel production: a critical review, Renew. Sust. Energ. Rev. 78 (2017) 356–368, https://doi.org/10.1016/j.rser.2017.04.110.
[18] E. Posadas Olmos, S. Bochon, M. Coca, M. García-González, P. García-Encina, R. Muñoz, Microalgae-based agro-industrial wastewater treatment: a preliminary screening of biodegradability, J. Appl. Phycol. 26 (2014), https://doi.org/10.1007/s10811-014-0263-0.
[19] A. Solovchenko, A.M. Verschoor, N.D. Jablonowski, L. Nedbal, Phosphorus from wastewater to crops: an alternative path involving microalgae, Biotechnol. Adv. 34 (2016) 550–564, https://doi.org/10.1016/j.biotechadv.2016.01.002.
[20] A. Hosseini, M. Shariati, Dunaliella biotechnology: methods and applications, J. Appl. Microbiol. (2009), https://doi.org/10.1111/j.1365-2672.2009.04153.x.
[21] M.J. Griffiths, S.T.L. Harrison, Lipid productivity as a key characteristic for choosing algal species for biodiesel production, J. Appl. Phycol. 21 (2009) 493–507, https://doi.org/10.1007/s10811-008-9392-7.
[22] G. Sibi, V. Shetty, K. Mokashi, Enhanced lipid productivity approaches in microalgae as an alternate for fossil fuels – a review, J. Energy Inst. 89 (2016) 330–334, https://doi.org/10.1016/j.joei.2015.03.008.
[23] T. Suganya, M. Varman, H.H. Masjuki, S. Renganathan, Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: a biorefinery approach, Renew. Sust. Energ. Rev. 55 (2016) 909–941.
[24] H. Tang, N. Abunasser, M.E.D. Garcia, M. Chen, K.Y. Simon Ng, S.O. Salley, Potential of microalgae oil from *Dunaliella tertiolecta* as a feedstock for biodiesel, Appl. Energy 88 (2011) 3324–3330.
[25] E.M. Fakhry, D.M.E. Maghraby, Fatty acids composition and biodiesel characterization of *Dunaliella salina*, J. Water Resour. Prot. 05 (2013) 894–899, https://doi.org/10.4236/jwarp.2013.59091.
[26] L. Clesceri, A. Greenberg, A. Eaton, Standard Methods for the Examination of Water and Wastewater, APHA, AWWA, WEF, (1998).
[27] Z. Wang, C. Benning, *Arabidopsis thaliana* polar glycerolipid profiling by thin layer chromatography (TLC) coupled with gas-liquid chromatography (GLC), J. Vis. Exp. (2011), https://doi.org/10.3791/2518.
[28] S. Van Wychen, K. Ramirez, L.M.L. Laurens, Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by in situ Transesterification: Laboratory Analytical Procedure (LAP), (2016), https://doi.org/10.2172/1118085.
[29] A.R. Sherwood, G.G. Presting, Universal primers amplify a 23s RDNA plastid marker in eukaryotic algae and cyanobacteria1, J. Phycol. 43 (2007) 605–608, https://doi.org/10.1111/j.1529-8817.2007.00341.x.
[30] S. Turner, K.M. Pryer, V.P. Miao, J.D. Palmer, Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis, J. Eukaryot. Microbiol. 46 (1999) 327–338.
[31] M.J. Atkinson, C. Bingman, Elemental Composition of Commercial Seasalts, (1996), p. 5.
[32] I. Moro, N. Rascio, N. La Rocca, M. Di Bella, C. Andreoli, *Cyanobacterium aponinum*, a new Cyanoprokaryote from the microbial mat of Euganean thermal springs (Padua, Italy), Algol. Stud. 123 (2007) 1–15, https://doi.org/10.1127/1864-1318/2007/0123-0001.
[33] D. Winckelmann, F. Bleeke, P. Bergmann, G. Klöck, Growth of cyanobacterium aponinum influenced by increasing salt concentrations and temperature, 3 Biotech. 5 (2015) 253–260, https://doi.org/10.1007/s13205-014-0224-y.
[34] S.E. Karatay, G. Dönmez, Microbial oil production from thermophile cyanobacteria for biodiesel production, Appl. Energy 88 (2011) 3632–3635, https://doi.org/10.1016/j.apenergy.2011.04.010.
[35] K. Milferstedt, W.C. Kuo-Dahab, C.S. Butler, J. Hamelin, A.S. Abouhend, K. Stauch-White, A. McNair, C. Watt, B.I. Carbajal-González, S. Dolan, C. Park, The importance of filamentous cyanobacteria in the development of oxygenic photogranules, Sci. Rep. 7 (2017), https://doi.org/10.1038/s41598-017-16614-9.
[36] X. Li, P. Přibyl, K. Bisova, S. Kawano, V. Cepák, V. Zachleder, M. Čížková, I. Brányiková, M. Vitova, The microalga *Parachlorella kessleri*—a novel highly efficient lipid producer the microalga, Biotechnol. Bioeng. 110 (2013), https://doi.org/10.1002/bit.24595.
[37] P. Přibyl, V. Cepák, V. Zachleder, Production of lipids in 10 strains of Chlorella and *Parachlorella*, and enhanced lipid productivity in Chlorella vulgaris, Appl. Microbiol. Biotechnol. 94 (2012) 549–561, https://doi.org/10.1007/s00253-012-3915-5.
[38] M.L. Bartley, W.J. Boeing, A.A. Corcoran, F.O. Holguin, T. Schaub, Effects of salinity on growth and lipid accumulation of biofuel microalga Nannochloropsis Salina and invading organisms, Biomass Bioenergy 54 (2013) 83–88, https://doi.org/10.1016/j.biombioe.2013.03.026.
[39] M. Töpel, M.I.M. Pinder, O.N. Johansson, O. Kourtchenko, A. Godhe, A.K. Clarke, Complete genome sequence of *Loktanella vestfoldensis* strain SMR4r, a novel strain isolated from a culture of the chain-forming diatom Skeletonema marinoi, Genome Announc. 6 (2018), https://doi.org/10.1128/genomeA.01558-17.
[40] I. Brettar, R. Christen, M.G. Höfle, *Aquiflexum balticum* gen. nov., sp. nov., a novel marine bacterium of the Cytophaga-Flavobacterium-Bacteroides group isolated from surface water of the central Baltic Sea, Int. J. Syst. Evol. Microbiol. 54 (2004) 2335–2341, https://doi.org/10.1099/ijs.0.63255-0.
[41] L.D. Zhu, Z.H. Li, E. Hiltunen, Strategies for lipid production improvement in microalgae as a biodiesel feedstock, Biomed. Res. Int. (2016), https://doi.org/10.1155/2016/8792548.
[42] S.M. Venkata, M.P. Devi, Salinity stress induced lipid synthesis to harness biodiesel during dual mode cultivation of mixotrophic microalgae, Bioresour. Technol. 165 (2014) 288–294, https://doi.org/10.1016/j.biortech.2014.02.103.
[43] L. Krienitz, E. Hegewald, D. Hepperle, V. Huss, T. Rohr, M. Wolf, Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae), Phycologia 43 (2004) 529–542, https://doi.org/10.2216/i0031-8884-43-5-529.1.
[44] F. Bücker, N.A. Santestevan, L.F. Roesch, R.J. Seminotti Jacques, M. do C.R. Peralba, F.A. de O. Camargo, F.M. Bento, Impact of biodiesel on biodeterioration of stored Brazilian diesel oil, Int. Biodeterior. Biodegrad. 65 (2011) 172–178, https://doi.org/10.1016/j.ibiod.2010.09.008.

[45] M. Chen, H. Tang, H. Ma, T.C. Holland, K.Y.S. Ng, S.O. Salley, Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*, Bioresour. Technol. 102 (2011) 1649–1655, https://doi.org/10.1016/j.biortech.2010.09.062.
[46] John Beardall, Mario Giordano, Acquisition and metabolism of inorganic nutrients by Dunaliella, Algae Dunaliella, 2009, pp. 173–188.
[47] J. Zhou, Y. Wen, Y. Wu, Effect of nitrogen and phosphorus ratio on algal growth in Lake Xuanwu, 5th Int. Conf. Bioinforma. Biomed. Eng. ICBBE 2011, 2011, pp. 1–4, , https://doi.org/10.1109/icbbe.2011.5780900.
[48] F. Lipmann, Metabolic generation and utilization of phosphate bond energy, Adv. Enzymol. Relat. Areas Mol. Biol, Wiley-Blackwell, 2006, pp. 99–162, , https://doi.org/10.1002/9780470122464.ch4.
[49] Y. Collos, P.J. Harrison, Acclimation and toxicity of high ammonium concentrations to unicellular algae, Mar. Pollut. Bull. 80 (2014) 8–23, https://doi.org/10.1016/j.marpolbul.2014.01.006.
[50] G. Markou, O. Depraetere, K. Muylaert, Effect of ammonia on the photosynthetic activity of Arthrospira and Chlorella: a study on chlorophyll fluorescence and electron transport, Algal Res. 16 (2016) 449–457, https://doi.org/10.1016/j.algal.2016.03.039.
[51] E. Eustance, R.D. Gardner, K.M. Moll, J. Menicucci, R. Gerlach, B.M. Peyton, Growth, nitrogen utilization and biodiesel potential for two chlorophytes grown on ammonium, nitrate or urea, J. Appl. Phycol. 25 (2013) 1663–1677, https://doi.org/10.1007/s10811-013-0008-5.
[52] J. Gutierrez, T. Kwan, J. Zimmerman, J. Peccia, Ammonia inhibition in oleaginous microalgae, Algal Res. 19 (2016) 123–127, https://doi.org/10.1016/j.algal.2016.07.016.
[53] K. Emerson, R.C. Russo, R.E. Lund, R.V. Thurston, Aqueous ammonia equilibrium calculations: effect of pH and temperature, J. Fish. Res. Board Can. (1975), https://doi.org/10.1139/f75-274.
[54] M. Maeda, O. Hisada, K. Ito, Y. Kinjo, Application of Pitzer's equations to dissociation constants of ammonium ion in lithium chloride–sodium chloride mixtures, J. Chem. Soc. Faraday Trans. 1 Phys. Chem. Condens. Phases 85 (1989) 2555–2562, https://doi.org/10.1039/F19898502555.